CHAPTER 10

VERATOX-AST TEST KIT

10.1 GENERAL INFORMATION

The Veratox AST test is a quick diagnostic tool to predict the presence of aflatoxin in corn and other commodities. The kit uses an enzyme-linked immunosorbent assay (ELISA) technique to obtain quantitative results from absorbance readings at 650 nm when sample readings are compared to a 20 ppb control and a pre-generated standard curve (0 to 400 ppb).

10.2 PREPARATION OF EXTRACTION SOLUTION

The extraction solvent used in the Veratox test method is a methanol/water (distilled or deionized) mixture consisting of 70 percent methanol (ACS grade or better) and 30 percent water.

a. Using a graduated cylinder, measure 700 ml of methanol and place it into a clean carboy with spigot.

b. Add 300 ml deionized or distilled water to the methanol and shake vigorously until it is completely mixed.

c. Label the container stating the mixture (70 percent methanol and 30 percent water), date of preparation, and initials of technician who prepared the solution.

d. Store this solution at room temperature in a tightly closed container until needed.

NOTE: To prepare smaller or larger amounts of solution use the ratio of 7 parts methanol to 3 parts of deionized or distilled water.

10.3 EXTRACTION PROCEDURES

a. Place a sheet of filter paper (Whatman 2V folded or S&S 24 cm pleated or equivalent) into a clean funnel mounted over a 25 X 200 mm (diameter x length) test tube or a collection beaker.

b. Label the collection container with the sample identification.

c. Place the 50-gram portion of the ground sample into the blender container.

d. Pour in 250 ml of the 70/30 percent methanol/water solution and securely close the blender top.

e. Blend for exactly two minutes at high speed.

f. Pour the resultant mixture from the blender into the funnel containing the filter paper and collect approximately 25 ml of extract.

10.4 TEST PROCEDURES

a. Preparation of Solutions.

(1) Place 3 ml of substrate (light green labeled bottle) solution into a clean, labeled reagent boat. Cover boat to protect solution from dust and light.

NOTE: Do not return any substrate solution to the original bottle once it has been removed.

(2) Place 3 ml of Red Stop (red labeled bottle) solution into a clean reagent boat. Cover boat to protect solution from dust and light.

b. Sample Analysis.

Do not use reagents or microwells from one kit serial number with reagents/wells from a different serial number. Reagent boats may be rinsed and reused.

(1) Open foil bag and remove 3 red-marked mixing wells for each sample to be tested (maximum of 4 samples or 12 wells). Place them in the microwell holder, and mark the left end of each strip with a "1."

Do not run more than four samples at one time.

(2) Remove 3 antibody wells for each sample to be tested (maximum of 4 samples or 12 wells). Place them in the microwell holder, and mark the left end of each strip with a "1."

(3) Reseal bag by folding over and tightly closing with a suitable fastener (large paper clip, tape, or suitable dust and light protectant).

(4) Place 100 µl of conjugate (blue-labeled bottle) into each mixing well using a 100 µl pipettor with a new tip. Prime the pipette tip first before dispensing the 100 µl. Discard the pipette tip.

NOTE: "Prime the pipette tip" is accomplished by drawing liquid up into the tip and dispensing it back into the bottle once or twice.

(5) Place 100 µl of control (yellow-labeled bottle) into the first mixing well labeled "1." Prime the tip before dispensing. If testing more than one sample, also place 100 µl of control into mixing well #4 for the second sample, mixing well #7 for the third sample, and mixing well #10 for the fourth sample. Discard the pipette tip.

(6) Place 100 µl of sample each in mixing wells #2 and #3. Prime the tip first before dispensing. Discard the tip. Subsequent samples should be placed in wells #5 and #6, then #8 and #9, and then #11 and #12.

See the diagram below for an example of the procedure.

	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	W11	W12
mixing wells	O	O	O	O	O	O	O	O	O	O	O	O
	C	S1	S1	C	S2	S2	C	S3	S3	C	S4	S4

"W" = well number (e.g., #1 through #12)
"C" = control
"S1, S2, S3, & S4" = sample numbers

(7) Using the 12 channel pipettor and the overfill method (see note below), mix the contents of the mixing wells by pipetting up and down in the tips 5 times.

NOTE: The "overfill method" is performed by drawing greater than 100 µl into the pipette tips by pressing the pipettor to the second stop before placing tips into the solution. Place tips into the liquid and release the plunger slowly and completely.

(8) Transfer 100 µl to the antibody coated wells (the unmarked, clear wells). To dispense only 100 µl, press plunger to the first stop.

(9) Mix in the antibody coated wells by gently sliding the microwell holder back and forth on a horizontal surface for 15 seconds. Be careful not to allow solution to splash out of wells.

(10) Immediately following mixing, incubate for 5 minutes. Discard all mixing (red marked) wells and tips.

(11) With a wash bottle containing deionized/distilled water, fill each antibody well and dump the contents into a waste receptacle. Repeat this step five times.

(12) Turn microwell holder, with wells in it, upside down on a paper towel and tap gently until water is removed from the wells.

(13) Using the 12 channel pipettor and the overfill method, place 100 µl of substrate into each well.

(14) Mix gently by sliding the microwell holder back and forth for 15 seconds on a horizontal surface for 15 seconds. Be careful not to allow solution to splash out of wells.

(15) Immediately following mixing, incubate for 5 minutes.

(16) Discharge the remaining substrate in the pipette tips by plunging once or twice without drawing any additional liquid up into the tips. Save these tips for the next step.

(17) Using the 12 channel pipettor and the overfill method, add 100 µl of the red stop solution (red labeled bottle) into each well.

(18) Mix gently by sliding the microwell holder back and forth for 15 seconds. Again be careful not to lose any solution from the wells. Visually check the appearance of the wells. Discard all pipette tips.

(19) Read in a microwell reader using a 650 nm filter within 5 minutes of the addition of the red stop solution.

c. Reading Results with Microwell Reader.

(1) Turn on the power to reader at the beginning of the test procedure to allow the electronics to stabilize. Make sure that the reader is properly attached to the computer.

(2) Turn on the computer and insert the VERATOX-AST software disc into the drive slot.

(3) Start the VERATOX program and select option "A" -"RUN AST."

(4) Check the kit identification and the standard curve values with the Standard Curve Program Calculated Points that came with the test kit. Edit standard or kit lot numbers as necessary.

(5) Press the "Enter" key, then press the "R" key to ready the computer to receive data from the microwell reader.

(6) Calibrate the microwell reader by following the instructions which appear on the LCD window of the reader.

(a) Remove sample carrier and press the "Enter" key.

(b) Place the filter holder in the W2 position and press "Enter." The instrument will calibrate on the W2 filter.

(c) Move the filter holder to the W1 position and press "Enter." The instrument will calibrate on the W1 filter.

NOTE: The Micro-well reader used in the official aflatoxin testing service is designed to do several testing functions. Each function requires specific set-up parameters. The required parameters for aflatoxin testing are: AF1 set up L S P, 12S, ABSORB, N Y N.@ To ensure that the Micro-well reader is properly set for aflatoxin testing, periodically check the display set-up as follows:

(d) Press the Display Set up. Display should read F1 setup L S P 12S ABSORB N Y N.

If the display reads differently, contact the Neogen Corporation representative for instruction. Otherwise, press the Display Set up again. This will return the instrument to normal operational mode.

(e) Press the "Clear" key, then the "Blank" key. This will blank the instrument on air and it is now ready to measure absorbance.

(f) Place the wells into the reader's sample holder. Make sure that the well marked "1" is in the far left position in the holder.

(g) Move the holder to the left so that the first well is under the reader and press the "Read" key. Repeat this process until all wells are read.

(h) Follow the instructions as requested by the software. Values displayed on the computer screen will be the mean of the duplicate measurements.

d. Troubleshooting.

The Veratox AST quantitative test requires two duplicate portions of a sample to be run in addition to a control portion. The mean OD reading of the duplicate portions is compared with the calculated OD points in the standard curve program to obtain a result in ppb. If the coefficient of variance (CV) between the OD values of the test portions is not within the required 15 percent range of difference, a "CV overrange@ error will be displayed as the test result. Typically, a "CV overrange@ message indicates that the test results are not valid and that another portion must be tested.

If the OD readings of the duplicate portions both indicate that the aflatoxin level is less than 300 ppb and a "CV overrange@ message appears, then an additional sample portion must be tested until the OD values of duplicate portions are within 15 percent of the mean OD and the "CV overrange@ message is eliminated. If the OD readings for the duplicate portions indicate that one of the portions exceeds 300 ppb and the other portion is less than 300 ppb and a "CV overrange@ message is displayed, the sample must be retested (full strength or diluted portion) until both of the OD readings are within the 15 percent range.

If the OD readings for the duplicate portions both indicate that the aflatoxin content exceeds 300 ppb but a "CV overrange@ message appears, then:

(1) an additional sample portion (diluted) must be tested until the OD values of duplicate portions are within 15 percent of the mean OD,

or

(2) official personnel may stop the testing if the applicant requests only a certification statement that aflatoxin exceeds 300 ppb. In this instance, official personnel will certificate the aflatoxin testing results as a "Aflatoxin exceeds 300 ppb.@

10.5 REPORTING AND CERTIFYING TEST RESULTS

a. Report all results on the pan ticket and the inspection log to the nearest whole ppb.

b. Sample results over 300 ppb are reported as >300 ppb unless a supplemental analysis is performed.

c. Refer to the Certification section of the handbook for more detailed certification procedures.

10.6 SUPPLEMENTAL ANALYSIS

a. Diluting the Sample Extract.

If quantitative results are above the test method's conformance limit, test results are reported as exceeding the limit. To determine and report an aflatoxin level higher than 300 ppb, the sample extract must be diluted so that a value between 5 and 300 ppb is obtained.

The final aflatoxin concentration is calculated by multiplying the results with the diluted extract by the dilution factor.

b. Example.

If the original analysis reported the aflatoxin value at 700 ppb, the sample extract would be diluted using the following procedures in order to obtain a true value.

(1) Dilute 5 ml of the original extract with 10 ml of the extraction solvent mixture. The total volume is 15 ml. This is a 1 to 3 dilution (compares volume in the beginning with the total volume in the end).

(2) Multiply the analytical results obtained by 3 to obtain the actual aflatoxin concentration. For example, if 240 ppb was the original value obtained with the diluted extract, the actual concentration in the original sample was 720 ppb.

$$\text{True Aflatoxin Value} = \frac{\text{Total Volume}}{\text{Initial Extract Volume}} \times \text{Aflatoxin Result}$$

$$\text{True Aflatoxin Value} = (15 \div 5) \times 240 \text{ ppb}$$
$$= 3 \times 240 \text{ ppb} = 720 \text{ ppb}$$

10.7 CLEANING LABWARE

a. Negative Tests (# 20 ppb).

(1) Labware.

Prepare a solution consisting of dishwashing liquid and water. Completely submerge the used glassware, funnels, beakers, etc., wash thoroughly, then rinse with clean water before reusing.

(2) Disposable Materials.

Place materials in a garbage bag for routine trash disposal.

b. Positive Tests (> 20 ppb).

(1) Labware.

Prepare a bleach solution consisting of 1 part bleach to 10 parts water (e.g., 100 ml bleach to 1,000 ml water). Completely submerge the used glassware, funnels, beakers, etc., and soak for at least 5 minutes. Remove items from the bleach/water solution, submerge in a dishwashing liquid/water solution, wash thoroughly, then rinse with clean water before reusing.

(2) Disposable Materials.

Prepare a bleach solution consisting of 1 part bleach to 10 parts water in a plastic pail labeled "bleach solution." Soak disposable materials, such as used columns, cuvettes, vials, test kit components, etc., for at least 5 minutes. Pour off the liquid down the drain and place the materials in a garbage bag and discard.

10.8 WASTE DISPOSAL

a. Negative Results (# 20 ppb).

If the test result is negative (equal to or less than 20 ppb), discard the filter paper and its contents (ground material) into a plastic garbage bag for disposal. Dispose of any remaining liquid filtrate in the chemical waste container.

b. Positive Results (> 20 ppb).

If the result is positive (more than 20 ppb), the ground portion remaining in the filter paper must be decontaminated prior to disposal. After disposing of the remaining filtered extract in the chemical waste container, filter approximately 50 ml of bleach through the filter containing the ground portion and allow to drain. Discard the filter paper and its contents (ground portion) into a plastic garbage bag for disposal. The bleach rinse filtrate collected may be treated as a non-hazardous solution and disposed of by pouring down the drain.

10.9 EQUIPMENT AND SUPPLIES

a. Materials Supplied in Test Kits.

(1) 48 antibody-coated wells.

(2) 48 red-marked mixing wells.

(3) 1 yellow-labeled bottle of 1.5 ml 20 ppb aflatoxin control.

(4) 1 blue-labeled bottle of 7 ml aflatoxin-HRP conjugate solution.

(5) 1 green-labeled bottle of 24 ml K-blue substrate solution.

(6) 1 red-labeled bottle of 32ml red stop solution.

b. Materials Required but not Provided.

(1) Methanol - ACS grade or better.

(2) Deionized or distilled water.

(3) 250 ml graduated cylinder.

(4) Whatman 2V folded or S&S 24 cm pleated (or equivalent) filter paper.

(5) Filter funnel.

(6) Sample collection tubes.

(7) Blender with mixing jars.

(8) Balance.

(9) Sample grinder.

(10) Bio Tek EL 301 Microwell strip reader with 650 nm filter.

(11) 12-channel pipettor.

(12) 100 µl pipet.

(13) Pipette tips.

(14) Microwell holder.

(15) Waterproof marker.

(16) 2 reagent boats (to hold substrate and red stop solutions).

(17) Timer.

10.10 STORAGE CONDITIONS

a. The kit is packaged in a sealed "foil bag" with a label indicating the lot number and expiration date.

b. Store test kits between 36°- 46°F when not in use. Avoid prolonged storage of kits at room temperature. Do not freeze test kits.

c. Bring kits up to room temperature 64°- 86°F prior to use.

d. Do not use kit components beyond their expiration date.